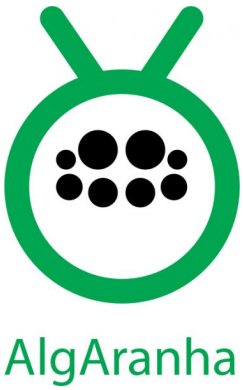
Gathering of expression vector units



Summary

September

- Given the fact that we were able to clone our 5' cassette, we were driven by a self-commitment to iGEM community that motivate us to spread this cassette worldwide. Consequently, we requested primers to assembly each part into iGEM backbone.

- For PCR purposes, BBa_K2136010 was used as template to amplify BBa_K2136013, BBa_K2136014. pUC19 plasmid was also used for this goal.

- Another caveat that made us slip was a wrong pair of designed primers that shrink iGEM legal prefix.

October

- Cloning race to obtain everything in iGEM backbone.

- Meanwhile, we also transformed our expression vector in algae to certificate that is working and to characterize a codon-optimized fluorescent protein (mCherry BBa_K2136016). Further experiments on this matter could be checked on the "**Characterization of expression vector**" section.

- Last molecular biology experiments encompassed insert verification by colony PCR on microalgae. So far, we were not able to successfully detect the cloned part, however, characterization demonstrates fluorescence measures. PCR failure probably due to chlorophyll interference in PCR reaction.

----------09/01----------

PCR of pJP22 parts

Viviane

Master mix

Item	Volume for 1 reaction	Volume for “15 reactions”
CG buffer	2.5 uL	37.5 uL
dNTPs	0.250 uL	3.75 uL
Foward primer	0.250 uL	-
Reverse primer	0.250 uL	-
Homemade polymerase	1 uL	15 uL
Betaine 5M	2.5 uL	37.5 uL
H2O	5.25 uL	78.75 uL
Template DNA	0.5 uL	-
Total	12.5 uL	172.5 uL

Primers x Template	Digested pJp22 with XhoI and BamHI	pJp22 with MV-L (from the ligation attempt)	pJp22 mCherry
Promoter primers (53 and 55)	P1	P2	P3
Resistance (Ble) primers (56 and 57)	R1	R2	R3
Terminator primers (58 and 59	T1	T2	T3

Promoter: ~680 bp

Resistance (ble): ~870 bp

Terminator: ~490 bp

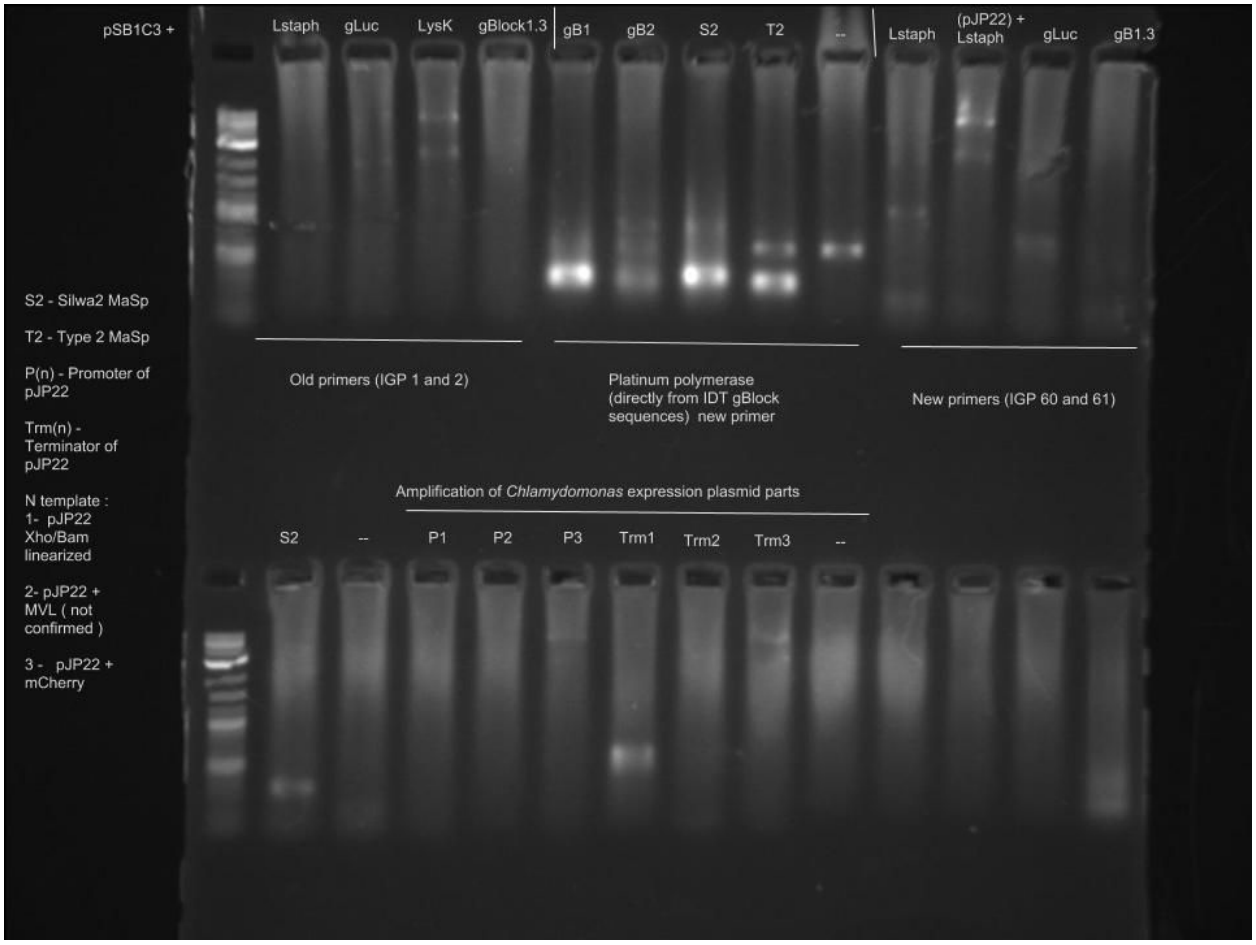
STEP	TEMP	TIME
Initial Denaturation	98°C	1 min
35 Cycles	98°C 60°C 72°C	15 seconds 30 seconds 45 seconds
Final Extension	72°C	5 minutes
Hold	4°C	

----------09/02----------

Gel electrophoresis of pJP22 parts (PCR)

Tiago, Viviane, Brayan

Terminator (T1) has a band in the desired size.



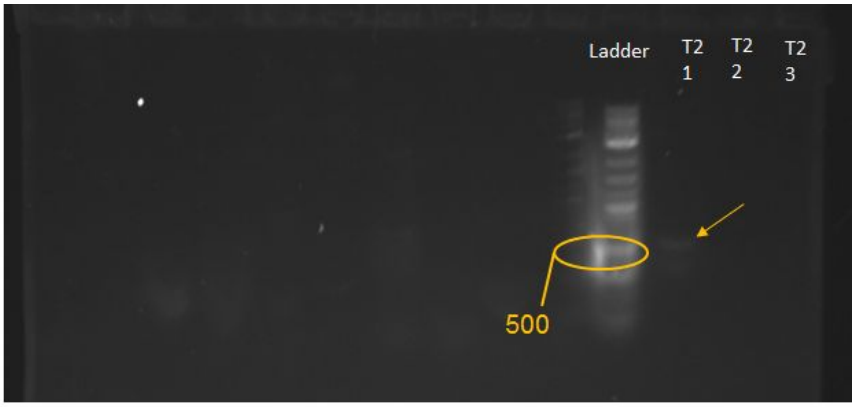
----------09/03----------

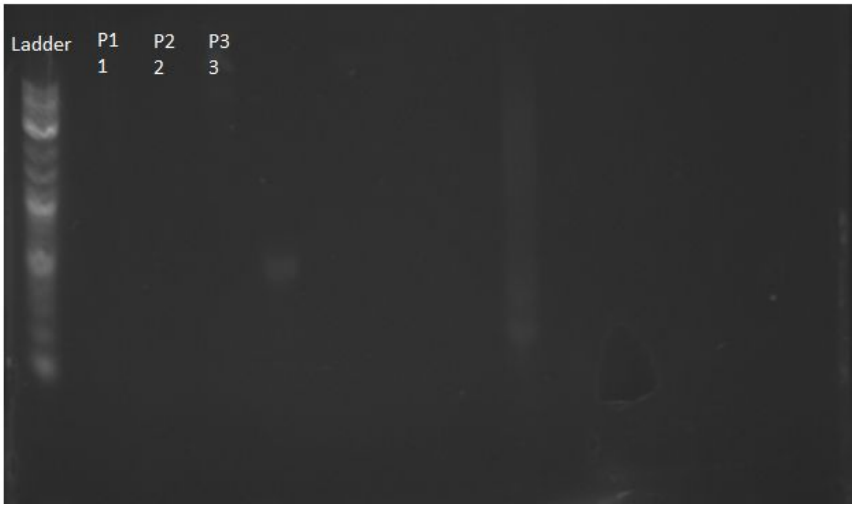
Gel electrophoresis of pJP22 parts (PCR)

Brayan

Confirming previous gel of 09/02. Terminator (T1) has a band in the desired size.

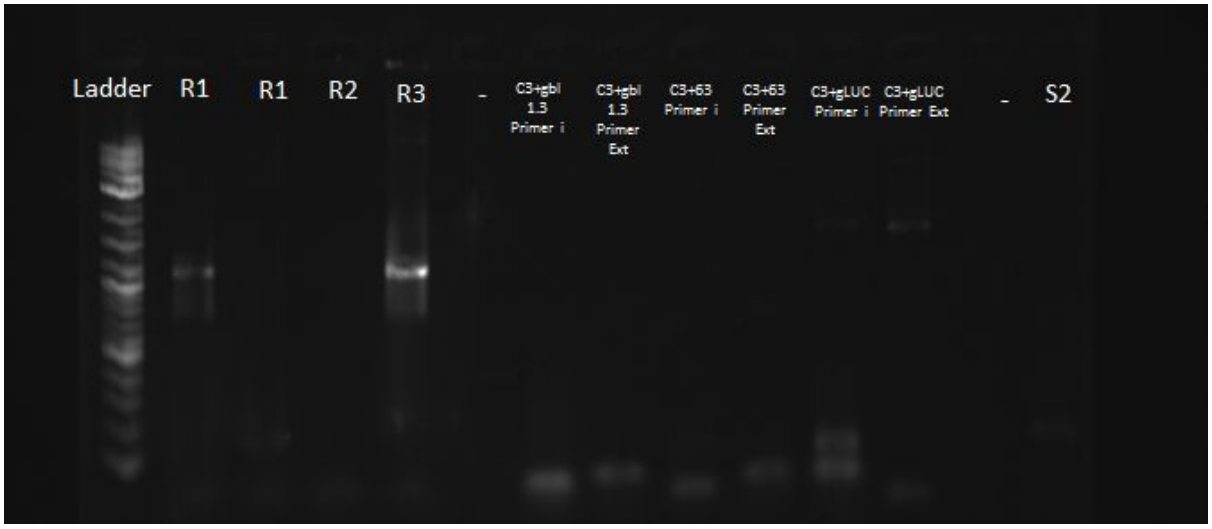
1 = pJP22 Xho/BamHI digested (29ng/ul ~)
2 = pJP22 + MVL (15ng/ul)
3 = pJP22 + mCherry (>100ng/ul)

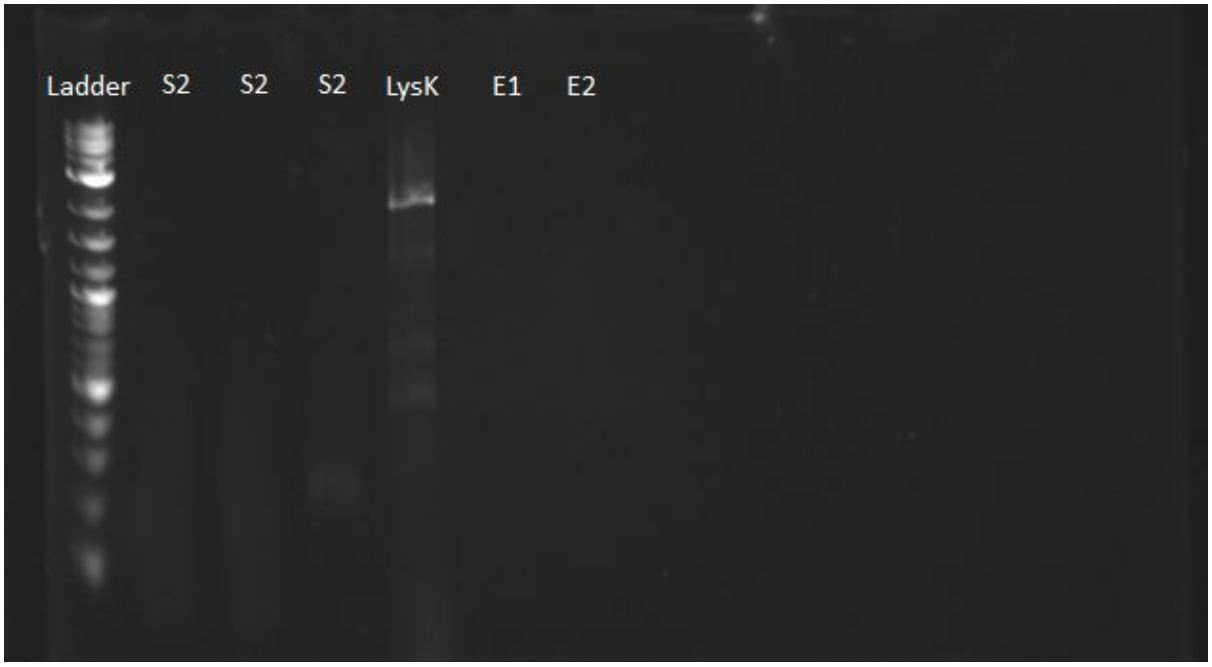




Gel electrophoresis of pJP22 parts (PCR)
Viviane

Resistance gene from pJP22 is working with both template 1 and 3.
Terminator gene from pJP22 is working with template 1.
No promoter region amplified.





----------09/05----------

PCR of pJP22 parts

Viviane

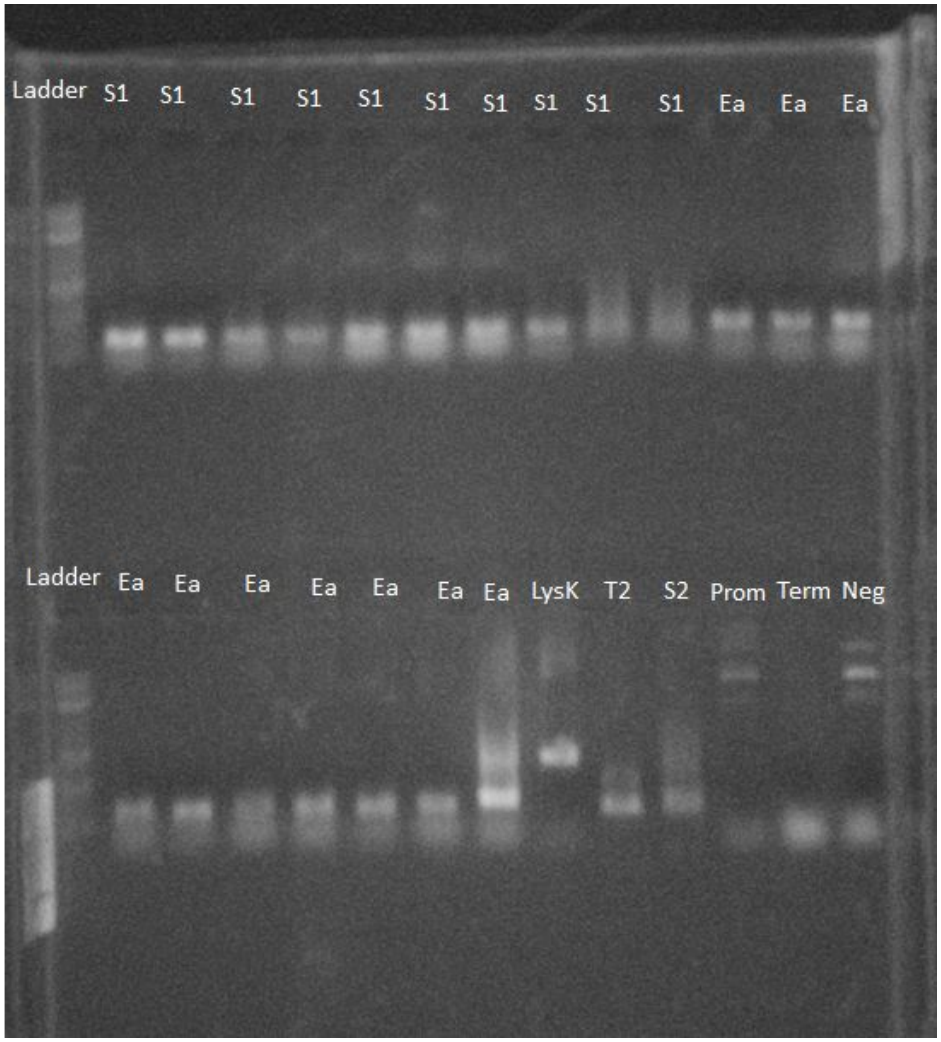
It was an attempt to amplify P1 and R3 of 09/01 to bind to pSB1C3.

Resistance primers: 56 and 57
Terminator primers: 58 and 59

Item	Volume for 1 reaction	Volume for 3 reactions
CG buffer	10 uL	30 uL
dNTPs	2.5 uL	7.5 uL
Foward primer	2.5 uL	-
Reverse primer	2.5 uL	-
Homemade polymerase	2.5 uL	7.5 uL
Betaine 5M	10 uL	30 uL
H2O	18 uL	54 uL
Template DNA	2 uL	-
Total	50 uL	150 uL

Gel electrophoresis of pJP22 parts (PCR)
Viviane

pJP22 parts were not amplified correctly.



----------09/10----------

PCR of pJP22 parts
Viviane

It is new attempt to amplify P1 and R3 of 09/01 to bind to pSB1C3.

Resistance primers: 56 and 57
Terminator primers: 58 and 59

Item	Volume for 1 reaction	Volume for 3 reactions
CG buffer	5 uL	15 uL
dNTPs	0.5 uL	1.5 uL
Foward primer	0.5 uL	-
Reverse primer	0.5 uL	-
Homemade polymerase	2 uL	6 uL
Betaine 5M	5 uL	15 uL
H2O	10.5 uL	31.5 uL
Template DNA	1 uL	-
Total	25 uL	75 uL

After the mix were prepared, I decided to divide the negative control at 2 tubes with 12.5 uL to test the PCR of promoter again (it failed at 09/01). Then, I added DNA template (pJp22 mCherry => P3) and its primers as described below.

Promoter primers: 53 and 55

Item	Volume for 1 reaction
CG buffer	2.5 uL
dNTPs	0.250 uL
Foward primer	0.250 uL
Reverse primer	0.250 uL
Homemade polymerase	1 uL

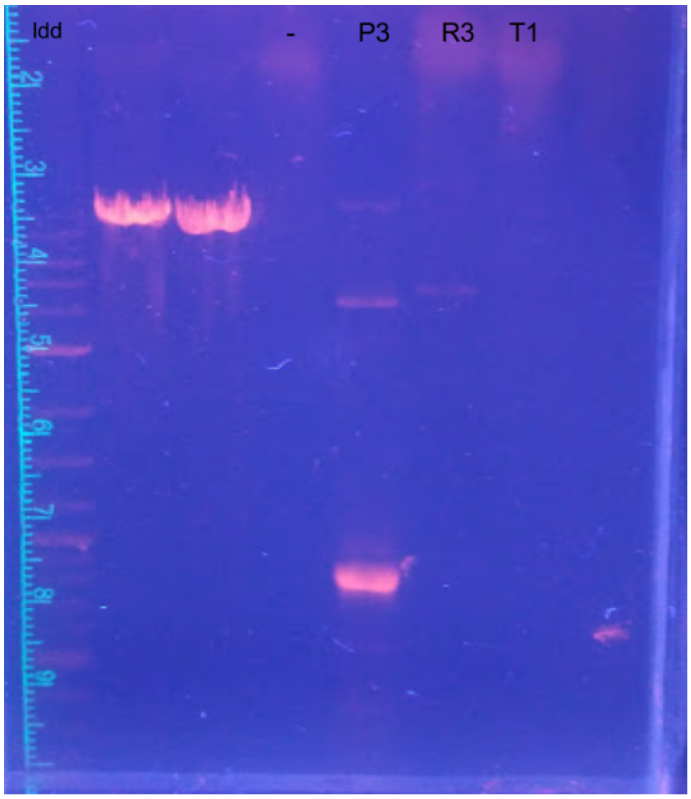
Betaine 5M	2.5 uL
H2O	5.25 uL
Template DNA	0.5 uL
Total	12.5 uL

----------09/12----------

Gel electrophoresis of pJP22 parts (PCR)

Viviane

This PCR was done at 09/10. P3 was amplified, R3 and T1 were not amplified.
Note: I think I put 1 µL of template at P3, not 0.5 µL like I usually do => repeat!



----------09/13----------

PCR of pJP22 parts

Viviane

The ideia here is to obtain the parts in large quantity to bind with pSB1C3. The templates were chosen according to previous PCRs that work. Some of the samples were placed on a different thermocycler to find out if there is some difference.

Item	Volume for 1 reaction	Volume for 20 reactions
CG buffer	2.5 µL	50 µL
dNTPs	0.250 µL	5 µL
Foward primer	0.250 µL	-
Reverse primer	0.250 µL	-
Homemade polymerase	1 µL	20 µL
Betaine 5M	2.5 µL	50 µL
H2O	5.25 µL	105 µL
Template DNA	0.5 µL	-
Total	12.5 µL	230 µL

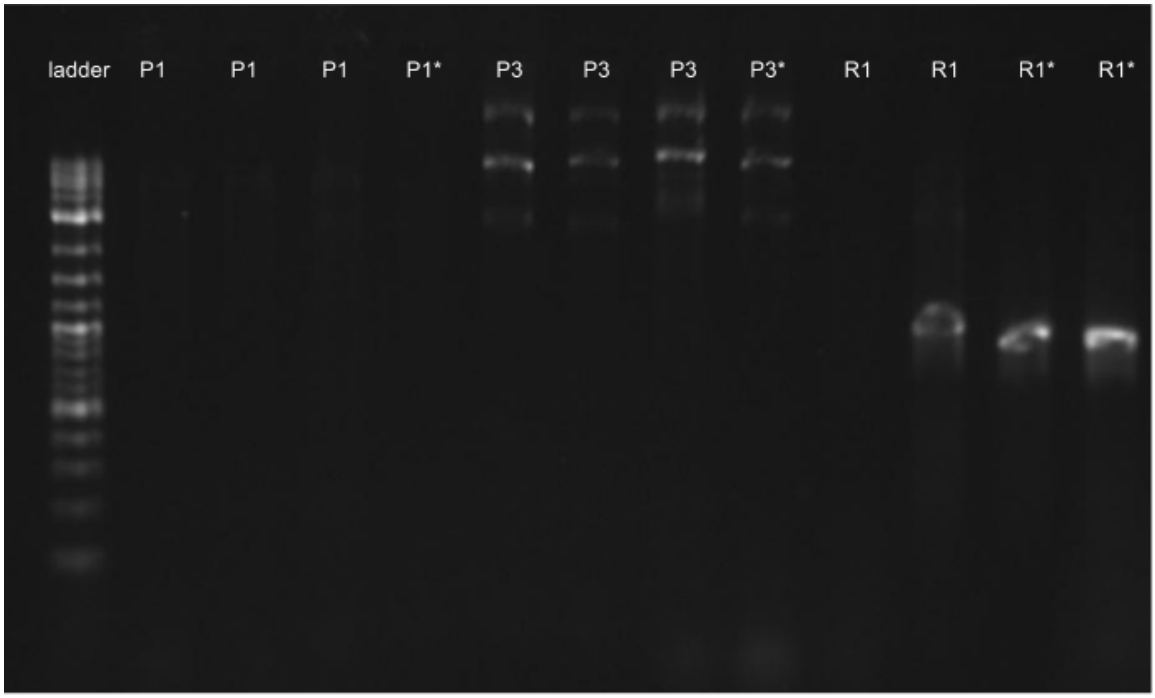
4x P1 (12.5 µL)
4x P3 (12.5 µL)
4x R1 (12.5 µL)
4x R3 (12.5 µL)
4x T1 (12.5 µL)

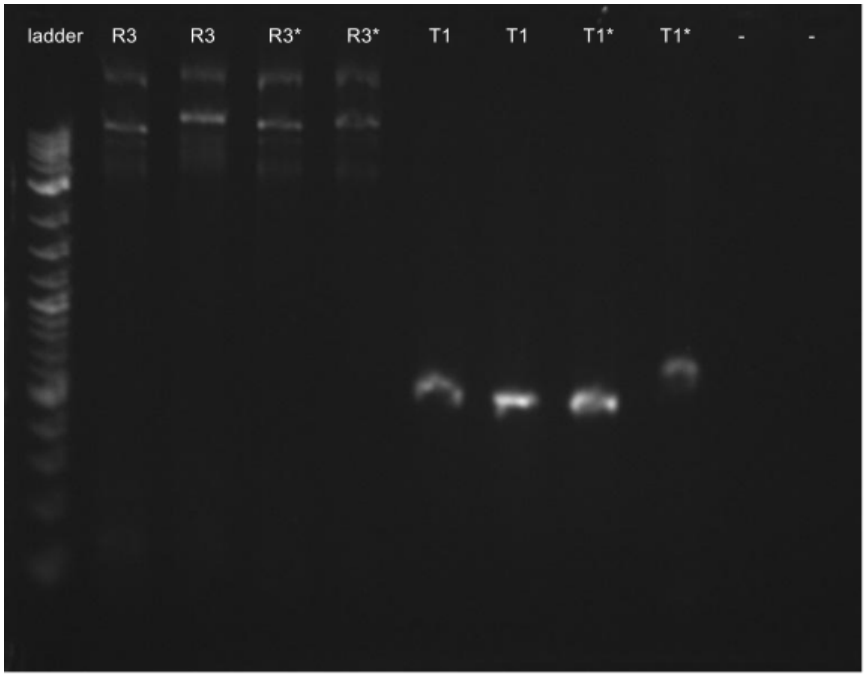
----------09/14----------

Gel electrophoresis of pJP22 parts (PCR)

Viviane

The promoter was not amplified like at 09/12. Resistance and terminator were amplified. I think I forgot to put primer at the first lane of R1.





(*): different thermocycler

----------09/16----------

Digestion

Viviane

All positive PCR of 09/14 were digested with EcoRI & PstI, it includes R1 and T1. João suggested do not purify PCR before digestion because we got a little quantity of DNA, then I did it. I put enzymes and buffer in the PCR reaction.

EcoRI	0.5 µL
PstI	0.5 µL
Buffer 2.1	1 µL
PCR reaction	~8.5 µL
Total	11.5 µL

----------09/17----------

PCR of pJP22 parts

Viviane

Its a another attempt of to amplify the promoter. The template is pJP22 digested with BamHI and XhoI, so I named it P3, although, it is not the same tube of 09/12.

Item	Volume for 1 reaction
Q5 master mix 2X (NEB)	12.5 µL
Foward primer	1.25 µL
Reverse primer	1.25 µL
H2O	10 µL
Template DNA	1 µL
Total	25 µL

STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds
35 Cycles	98°C 65°C 72°C	10 seconds 30 seconds 5 minutes (ops)
Final Extension	72°C	2 minutes
Hold	4°C	

----------09/18----------

PCR of pJP22 parts

Viviane and Brayan

It is an attempt to reutilize previous failed PCR reactions (09/14), same tubes were used for a new PCR cycling. It was taken into account the addition of template (0.5 µL) and polymerase (0.5 µL).

Thought: Touch-down cycling for recovering every kind of template and 44°C to guarantee that everything binds to the template.

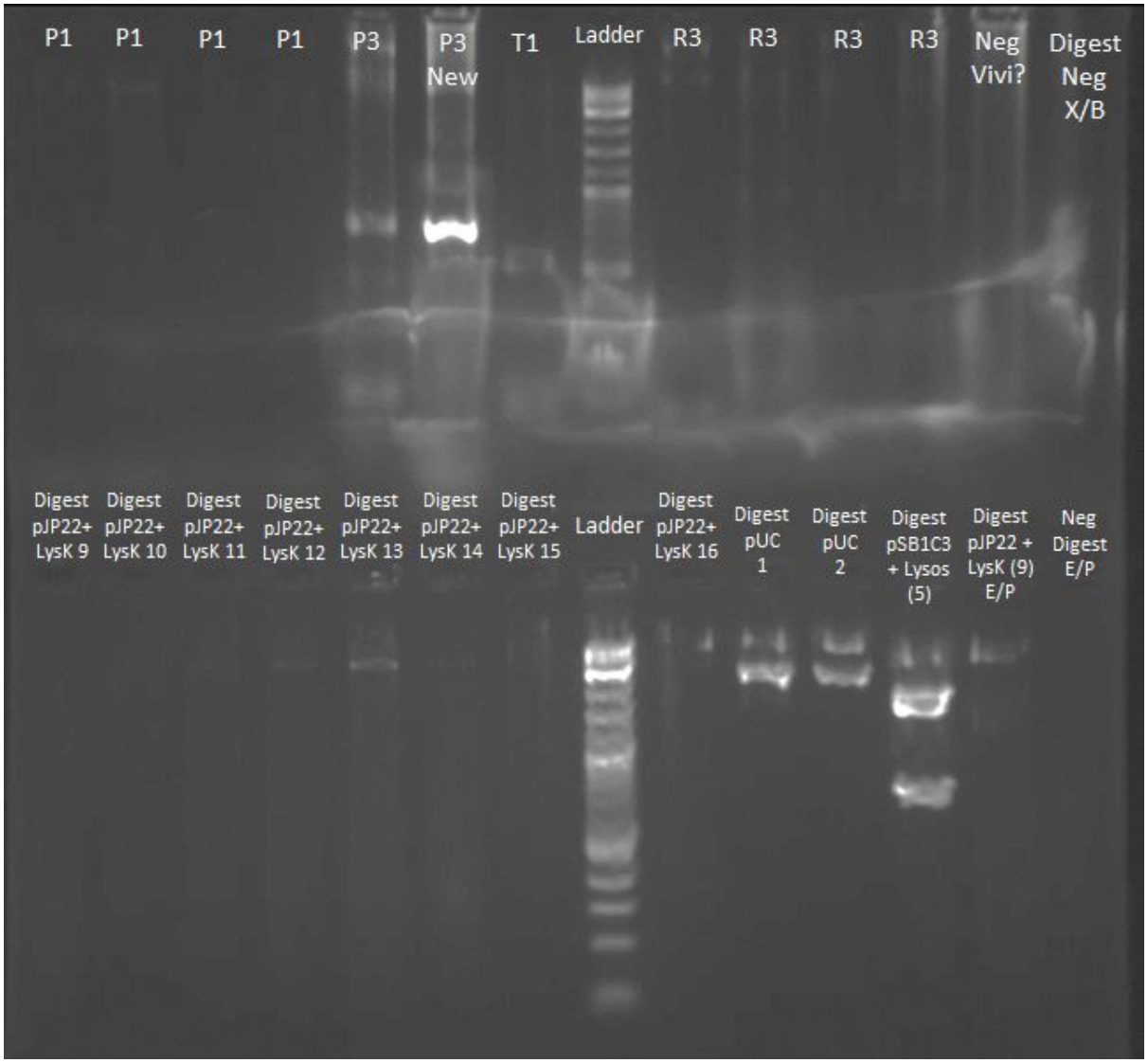
STEP	TEMP	TIME
Initial Denaturation	98°C	1 min
Touch Down Cycling	From 64°C to 56°C (Annealing temperature)	30 seconds
44°C annealing condition	44°C	30 seconds
22 Cycles	98°C 60°C 72°C	10 seconds 30 seconds 30 seconds
Final Extension	72°C	2 minutes
Hold	4°C	For ever

----------09/20----------

Gel electrophoresis of pJP22 parts (PCR)

Brayan

Promoter and resistance PCRs from 09/17 made by Viviane.



PCR of pJP22 parts
Viviane

PCR of promoter (P3) using Q5 performed as expected at 09/17, so I repeated it once again and I did it for resistance gene too. Now, the pcr of resistance gene was done with gBlock 1 as template (Rgb1) because it does not have an undesirable restriction site like pJP22.

Item	Volume for 5 reactions of each one
Q5 master mix 2X (NEB)	62.5 µL
Foward primer	6.25 µL
Reverse primer	6.25 µL
H2O	50 µL
Template DNA	-
Total	125 µL

5x P3 (25 µL)
5x Rgb1 (25 µL)

STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds
35 Cycles	98°C 65°C 72°C	10 seconds 30 seconds 30 seconds
Final Extension	72°C	2 minutes
Hold	4°C	

Preparation of C. reinhardtii inoculum
Tiago and João

Stock strains of microalgae (cc1610 and cc1010) were picked from stored plates (that supply actually lab's stock) and transferred into a 200-ml Tris-Acetate-Phosphate (TAP) culture on a 500 ml flask.

Inoculum is put on a shaker at 250 rpm adapted with proper illumination system.

----------09/21----------

Digestion of pJP22 + mCherry
Tiago and Joao

Preparation of expression vector is done by releasing all the expression cassette which is flanked by XbaI and KpnI.

Item	Rx
Cutsmart	5
XbaI	2.0 ul
KpnI	2.0 ul
Vector	40 ul
H_2O	Qsf 50 ul
Total	50ul

To guarantee total digestion, reaction is leaved 8 hours or more at 37°C on thermocycler.

----------09/22----------

Column purification of digested insert
Viviane
After overnight digestion, column purification was done and immediately quantified

----------09/23----------

Electroporation of prepared expression plasmid on C. reinhardtii

Tiago and Joao

Similar to bacterial transformation, microalgae must reach a certain growth state which allow it to embody and recombine digested insert, which is actually the whole expression cassette.

Assessing the growing state of algae was standardized by measuring optical density at 750nm. OD should not pass 0,3 so as to have a reasonable transformation efficiency. This is mainly recommended due to the final number of cells per milliliter, which is expected to be $3x10^6$.

For microalgae electroporation, it's needed at least 500 ng of digested insert. Maximum volume allowed to transform is 10 ul.

According to the final volume of inoculum (200ml), we distributed it on proper amount of 50-ml falcons for pelleting. Based upon this volume, it's estimated that 100 ml can yield up to 4 potential transformant cells.

Once pelleted, all further steps must be done on hood. Carefully, remove all TAP supernatant and resuspend cells in 1ml of sterile water. Take into consideration that cell final concentration once centrifuged is 50-fold higher (if start volume of distributed inoculum was 50 ml).

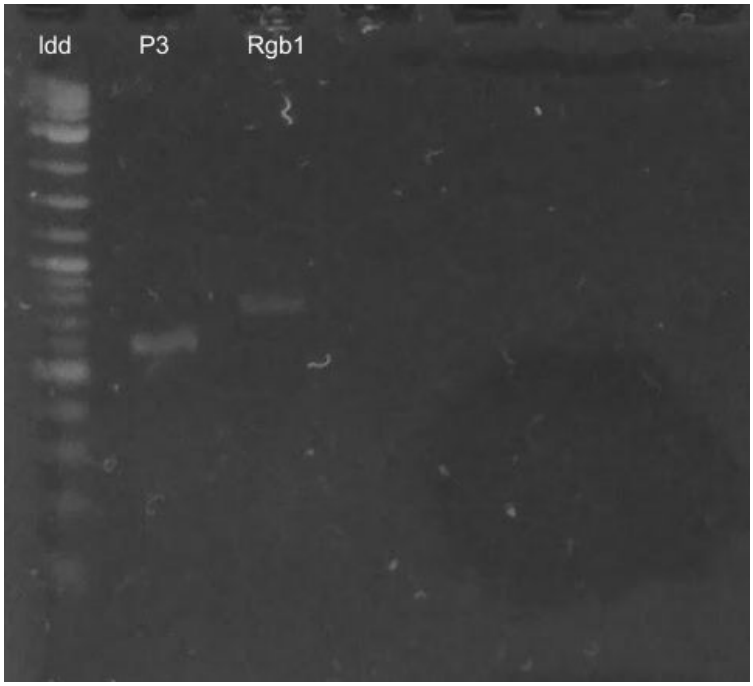
Immediately after, 250 ul of resuspended cells is pipetted on each electroporation cuvette (0,2 mm). Suitable amount of plasmid (500 ng) is also added into reaction. Incubate on ice for 5 to 10 minutes. Electroporate under 800V. One critical point that can hamper electroporation itself came with over-carried salts from TAP culture, therefore, supernatant remotion must be done carefully as stated before.

Leave electroporated cells 15 minutes on recovery phase until pouring into a 10-ml TAP culture supplemented with sucrose. This culture should be incubated for 24 hours in constant agitation to allow proper and homogeneous growth.

----------09/24----------

Gel electrophoresis of pJP22 parts (PCR)
Viviane

Pcr of promoter and resistance sequences using Q5 was confirmed.



PCR purification
Viviane

Promoter and resistance sequences were purified.

Sample	Concentration
P3	274.9 ng/µL
Rgb1	181.7 ng/µL

Plating transformed microalgae on 09/23
Brayan

Pellet cells for 20 minutes at 3500 rpm. Cooling is no needed. 600 ul of each transformant (from a 10 ml-TAP sucrose culture) is fairly distributed into two TAP plates containing ampicilin and

antifungal solutions, and also zeocin for positive pressure selection of transformant bearing Ble-resistance gene (BBa_K2136014).

----------09/26----------

Digestion

Brayan

Two parts of ourdevice were digested for further ligation on C3 and pUC. Two additional plasmids were tested for final confirmation.

1. P3
2. Rgb1
3. pJP22 + LysK (9)
4. pJP22 + LysK (10)

Item	1, 2	3, 4
EcoRI	1 µL	0.5 µL (Thermo enzyme)
PstI	1 µL	0.5 µL (Thermo enzyme)
Buffer 2.1	2.9 µL	1.0 µL Buffer H (Thermo)
H2O	0	3 µL
DNA	24~25 µL	5 µl
Total	29.9 µL	10 µl

----------09/27----------

Ligation
Viviane

The values were based on the molecular weight of plasmids and inserts, so that their molecular proportion was 1:3.

Item	pSB1C3			pUC19		
	P3	Rgb1	T1	P3	Rgb1	T1
Vector (~50ng)	1.72 µL	1.72 µL	1.72 µL	1.1 µL	1.1 µL	1.1 µL
Insert	0.28 µL	0.20 µL	2.67 µL	0.22 µL	0.16 µL	2.05 µL
Buffer	1 µL	1 µL	1 µL	1 µL	1 µL	1 µL
T4 ligase	1 µL	1 µL	1 µL	1 µL	1 µL	1 µL
H2O	6 µL	6.08 µL	3.61 1 µL	6.68 1 µL	6.74 µL	4.85 µL
Total	10 µL	10 µL	10 µL	10 µL	10 µL	10 µL

Note: quantity of promoter and terminator were changed when I did, but it must not to be a problem.

Competent cells + transformation
Brayan

On-day electrocompetent cells were prepared in order to defy our standard method. This new method is based on http://www.nature.com/articles/srep24648. Briefly, it highlights the advantage of preparing your electrocompetents cell on the same day you gonna use and using room temperature (avoiding the fact that everything should be kept on ice or cold).

8 tubes with 60ul of cells were prepared

Viviane's ligations from the same day (**09/27**) were used for this test.
Selected plasmids:

C3+P3	C3+Rgb1	C3+T1	pUC+P3	pUC+Rgb1	pUC+T1

Also, 10pg/uL of C3 from transformation efficiency test (iGEM) and pJP22+Type 2 2-mer

----------09/28----------

Inoculum

Viviane

Some plates of Brayan have colonies.

pUC+P3	pUC+Rgb1	pUC+T1	pSB1C3+P3	pUC+Rgb1	pUC+T1	pSB1C3+T1

Digestion of pJP22 parts

Brayan

Every plasmid was quantified (see below) and was used for digestion in order to confirm insert presence.

Plasmid	Concentration	260nm-280nm ratio
pUC + P3 1	181,3	1,95
pUC + P3 2	144,3	1,89
pUC + P3 3	24,8	1,82
pUC + T1 1	33,3	1,82
pUC + T1 2	72,4	1,94
pUC + T1 3	37,8	1,89
pUC + Rgb1 1	78,4	2,01
pUC + Rgb1 2	89,2	1,94
pUC + Rgb1 3	22,5	1,91
C3+P3 1	22,9	1,95
C3+P3 2	25,0	2,14
C3+P3 1	12,8	1,83
C3+T1 1	24,8	2,00

C3+T1 2	14,2	2,08
C3+T1 3	33,8	2,03

----------09/30----------

Gel electrophoresis of digested plasmids

Brayan

pUC + P31	pUC + P32	pUC + P33	pUC + T11	pUC + T12	pUC + T13	pUC + R1	pUC + R2	Leakage	1kb plus	pUC + R3	Negative	C3 + P31	C3 + P32	C3 + P33
C3 + T11	C3+ T12	C3 + T13	1kb plus	mCh 1	mCh 2	mCh 3	mCh 4							

Result: Seems like one-side digestion was achieved, regardless the fact that it was supposed to be a double digestion. mCherry is ok.

Proceed to: Confirm by PCR.

Item	1Rx	10Rx
Buffer 2.1	1	10ul
Eco	1.0	10ul
Pst	1.0	10ul
DNA	50 (all reaction)	-
Total	60ul	600ul

PCR of pJP22 plasmids coming from 09/27
Viviane

Digestion seemed inconsistent to confirm presence of device parts, so I prepared a PCR reaction with rest of plasmids that were left after almost-total digestion

Item	Volume for 1 reaction
Q5 master mix 2X (NEB)	6.25 µL
Foward primer	0.625 µL
Reverse primer	0.625 µL
Template DNA + H20	5 µL
Total	12.5 µL

Digestion
Brayan

PCR products of mCherry were digested to bind with pSB1C3 and pUC.

Item	Reaction
Buffer 2.1	3.0 ul
EcoRI	1.0 ul
PstI	1.0 ul
DNA	25ul~ ul
Total	30 ul

PCR purification
Viviane

After digestion, PCR products of mCherry were column purified.
Quantification: around 100 ng/ul

Ligation
Viviane

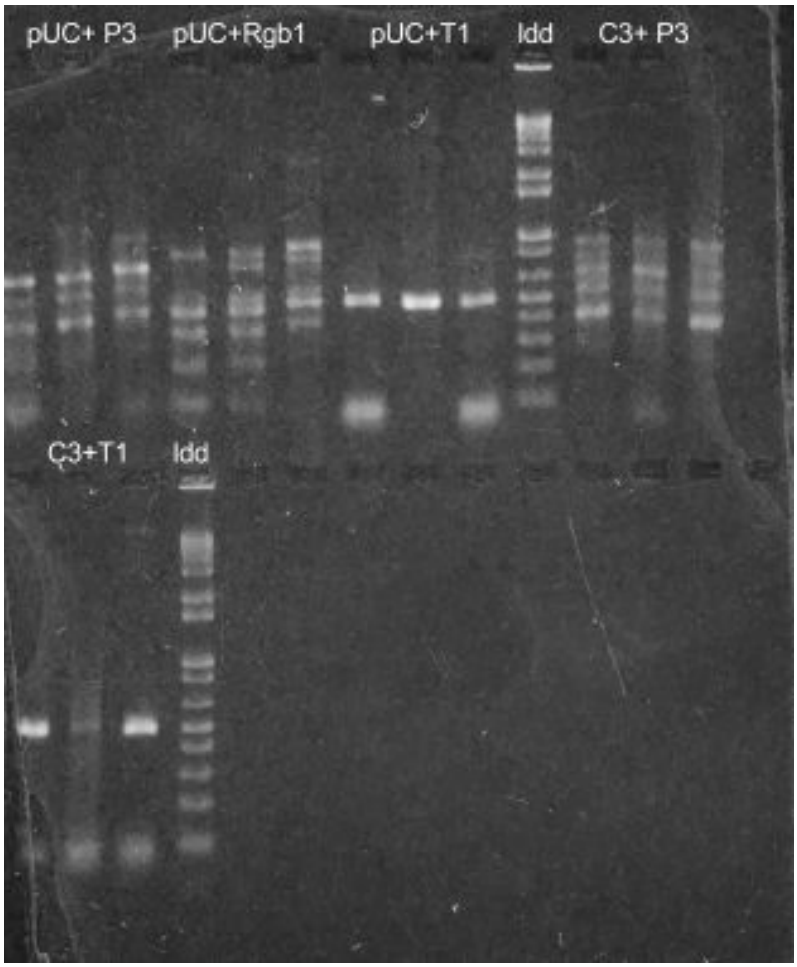
Item	pUC19	pSB1C3
	mcherry	
Vector (~50ng)	1 µL	5 µL
Insert	0.4 µL	0.5 µL
Buffer	1 µL	1 µL
T4 ligase	0.5 µL	0.5 µL
H2O	7.1 µL	3 µL
Total	10 µL	10 µL

----------10/02----------

Gel confirmation of pJP22 parts (PCR)

Brayan

pUC +P3 1	pUC +P3 2	pUC +P3 3	pUC+ Rgb1 1	pUC + Rgb 1 2	pUC + Rgb 1 3	pUC + T1 1	pUC + T1 2	pUC + T1 3	1kb ladd er plus	C3+ P3 1	C3+ P3 2	C3+ P3 3
C3+ T1 1	C3+ T1 2	C3+ T1 3	1kb ladde r plus									

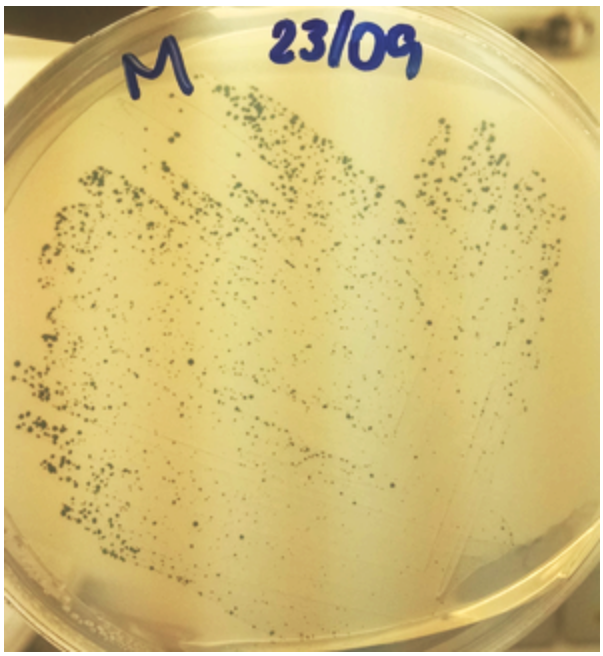


----------10/03----------

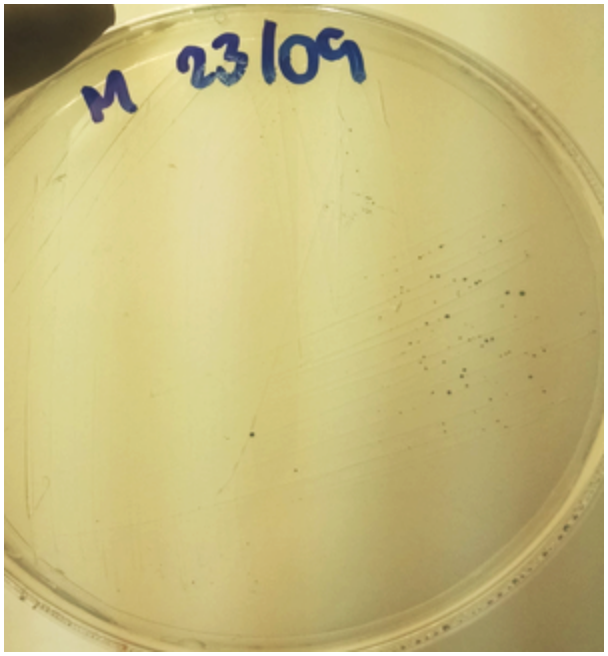
Colony Screening - pJP22+mCherry

João and Brayan

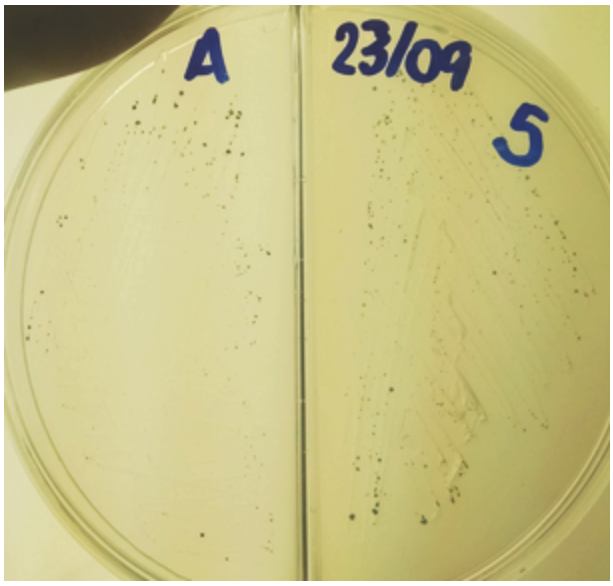
Cells transformed from 09/23.



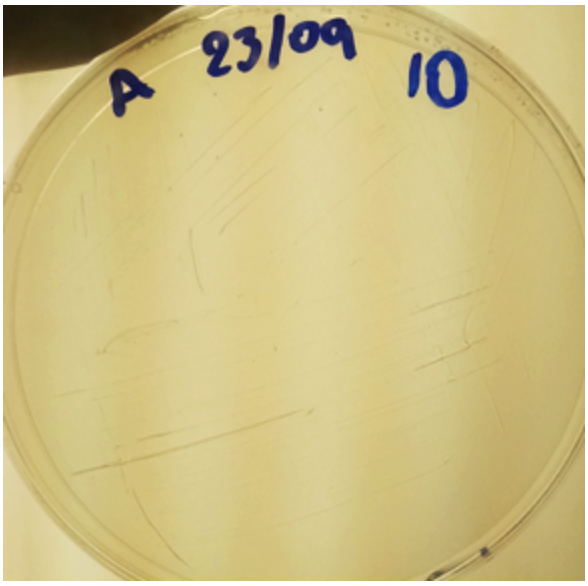
pJP22 mCherry transformants - Zeocin 5 ug/mL



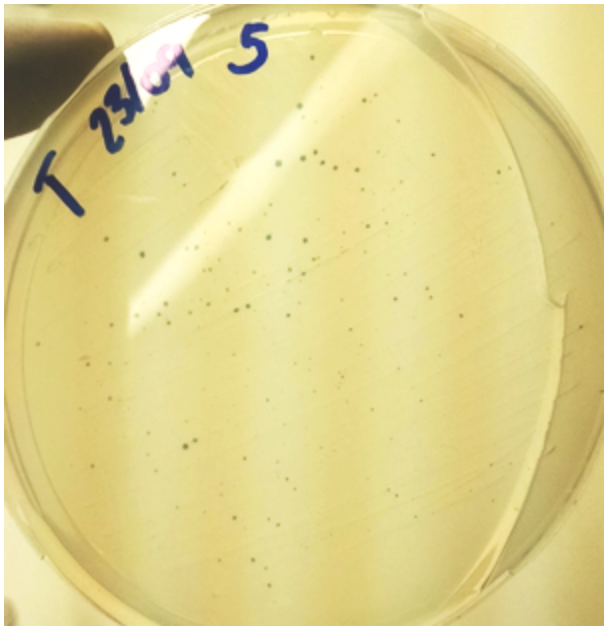
pJP22 mCherry transformants - Zeocin 10 ug/mL



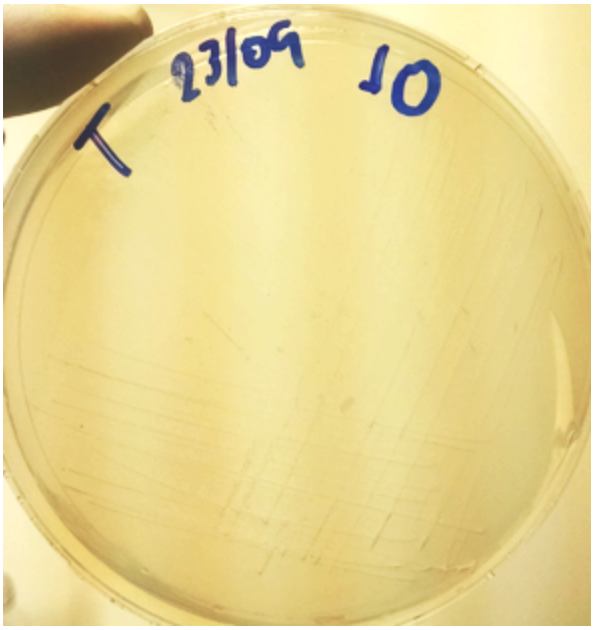
pJP22 mCherry transformants - Z5 (Water Transformed)



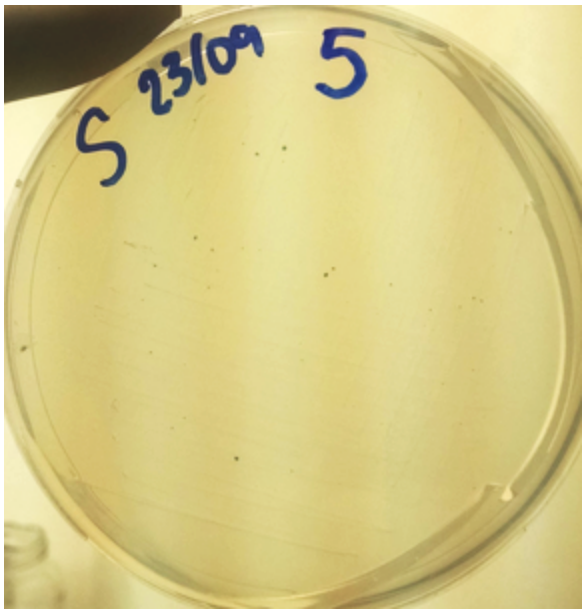
pJP22 mCherry transformants - Z10 (Water Transformed)



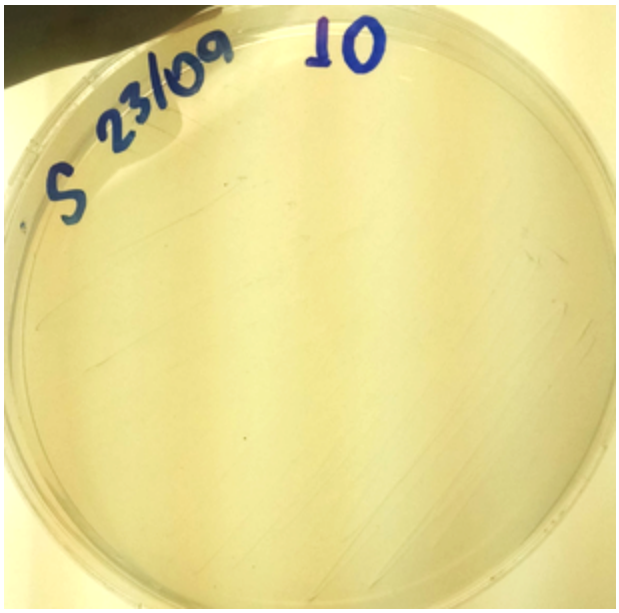
pJP22 mCherry transformants - Z5 (TAP Transformed)



pJP22 mCherry transformants - Z10 (TAP Transformed)



pJP22 mCherry transformants - Z5 (Sapphire Transformed)



pJP22 mCherry transformants - Z10 (Sapphire Transformed)

Screening by Colony picking and growing in a Black Clear Bottom 96 well plate.

200 uL TAP per Well
Agitation of 800 RPM
25ºC +-1ºC
80 µE m^{-2} s^{-1}
Clear sealing film

	1	2	3	4	5	6	7	8	9	10	11	12
A	Z10M	Z10M	Z10M	Z10M	Z10M	Z10M	Z10M	Z10M	Z10M	Z10M	Z10M	Z10M
B	Z5M	Z5M	Z5M	Z5M	Z5M	Z5M	Z5M	Z5M	Z5M	Z5M	Z5M	Z5M
C	Z10A	Z10A	Z10A	Z10A	Z5A	Z5A	Z5A	Z5A	Z5A	Z5A	Z5A	Z5A
D	Z5A	Z5A	Z5A	Z5A	Z5A	Z5A	Z5A	Z5A	Z5A	Z5A	Z5A	Z5A
E	Z10T	Z10T	Z10T	Z5T	Z5T	Z5T	Z5T	Z5T	Z5T	Z5T	Z5T	Z5T
F	Z5T	Z5T	Z5T	Z5T	Z5T	Z5T	Z5T	Z5T	Z5T	Z5T	Z5T	Z5T
G	Z5S	Z5S	Z5S	Z5S	Z5S	Z5S	Z5S	Z5S	Z5S	Z5S	Z5S	Z5S
H	CC1690 WildTyp	cc1690	cc1690	cc1690	cc1690	cc1690	TAP	TAP	TAP	mCherry	mCherry	mCherry

Z10M

Mode mCherry Reading	**Fluorescence Top Reading**	
Excitation Wavelength	**575**	**nm**
Emission Wavelength	**608**	**nm**
Excitation Bandwidth	**9**	**nm**
Emission Bandwidth	**20**	**nm**
Gain	**200**	**Manual**
Number of Flashes	**10**	
Integration Time	**20**	**µs**
Lag Time	**0**	**µs**

Settle Time	**0**	**ms**
Z-Position (Manual)	**18141**	**Mm**

Label: Label4

Mode - Density Chlamy	**Absorbance**	
Wavelength	**750**	**nm**
Bandwidth	**9**	**nm**
Number of Flashes	**25**	
Settle Time	**0**	**ms**

Start Time: **03/10/2016 21:17:06**

Label: Label3

Mode - Chlorophyll Reading	**Fluorescence Top Reading**	
Excitation Wavelength	**440**	**nm**
Emission Wavelength	**680**	**nm**
Excitation Bandwidth	**9**	**nm**
Emission Bandwidth	**20**	**nm**
Gain	**100**	**Manual**
Number of Flashes	**10**	
Integration Time	**20**	**µs**
Lag Time	**0**	**s**
Settle Time	**0**	**ms**

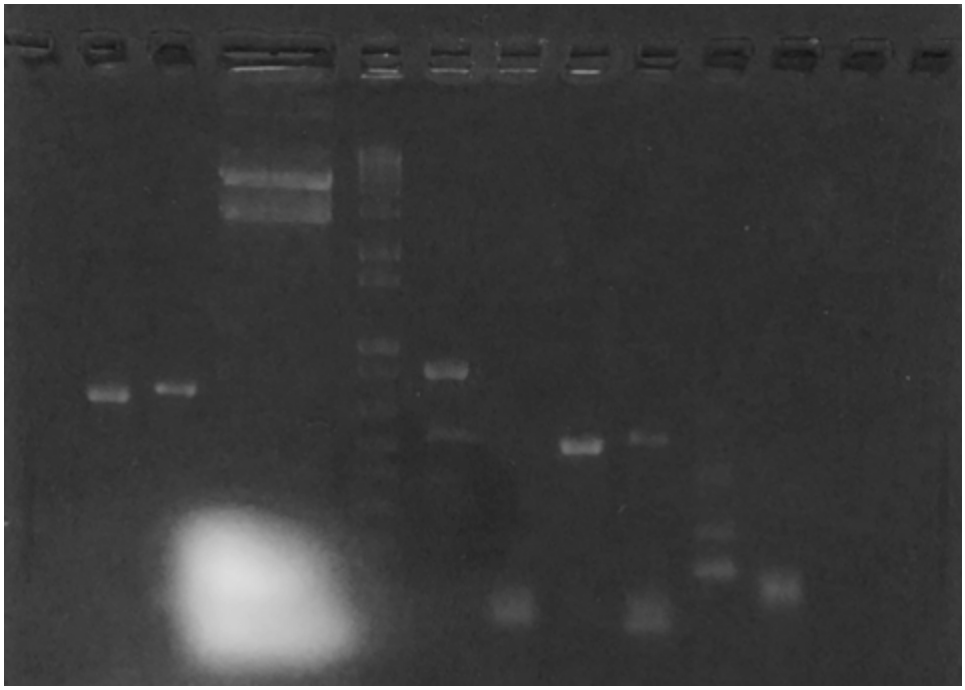
Z-Position (Manual) **18141** **µm**

Start Time: **03/10/2016 21:19:03**

----------10/04----------

PCR of pJP22 parts
Joao

Template
pJP22 type2 10/03
C3 gb1.3 09/13



	Promoter	Neg	USER	1kb ladder plus	Resistance	Neg1	Terminator	Neg	MaSp2	Neg			

Digestion
Viviane

	pSB1C3 (153 ng/µL)	Promoter (3x25 µL) Reistance (3x25 µL) Terminator (3x25 µL)
XbaI	1 µL	0.5 µL
SpeI	1 µL	0.5 µL
Cutsmart	5.17 µL	2.89 µL
H2O	0	0
DNA	45 µL	25 µL
Total	52.17 µL	28.89 µL

Item	Masp 2 (5x25 µL)
XhoI	0.5 µL
BamHI	0.5 µL
Cutsmart	2.89 µL
H2O	0
DNA	25 µL
Total	28.89 µL

----------10/06----------

PCR purification
Viviane

Promoter	129.5 ng/µL
Resistance	143.4 ng/µL

Terminator	86.8 ng/µL
Masp 2	90 ng/µL

Ligation
Joao and Fabio

USER	Promoter + C3	Resistance + C3	Terminator + C3	C3 negative

Two reactions were prepared for each ligation. One incubated at room temperature for 1 hour and the other one at 16°C overnight and heat inactivated afterwards.

Transformation
Joao & Fabio

This transformation was done following on-day cells for electroporation.

C3+Insert ligations from 20/08 batch	C3+Device parts purified from PCR purification made by Viviane	Negative C3

Results (next day): C3+Device parts grown but seems as contamination.
Recommendation: Re-do this parts with NEB cells.

Digestion of *C. reinhardtii* expression vector + mCherry
Brayan

Starting concentrations for each plasmid was:
A = 981,2 ng / ul
B = 594,1 ng / ul
C = 142,9 ng / ul

Item	pJP22 + mCherry (A)	pJP22 + mCherry (B)	pJP22 + mCherry (C)
Cutsmart	2,5	4,0	5,0
XbaI	2.0 ul	2.0 ul	1,0

KpnI	2.0 ul	2.0 ul	1,0
Vector	15 ul	30 ul	40 ul
Total	20 ul	40 ul	50 ul

----------10/10----------

New transformation on C. reinhardtii chassis
Brayan

Following last described protocol for microalgae electroporation, we performed once again a new transformation with the expression vector bearing mCherry.

These three digested vectors were transformed to guarantee mCherry results and further colony PCR of transformants.

pJP22 + mCherry (A)	pJP22 + mCherry (B)	pJP22 + mCherry (C)

----------10/11----------

Transformation of C3+expression vector parts on NEB cells
Brayan

Previously ligated sequences incubated overnight were used for this transformation

pSB1C3+Promoter	pSB1C3+Resistance	pSB1C3+Terminator	Negative pSB1C3

Colony PCR of likely clones
Brayan

Item	Volume for 1 reaction
Q5 master mix 2X (NEB)	6.25 µL
Forward primer	0.625 µL
Reverse primer	0.625 µL
Template DNA + H20	5 µL

Total	12.5 µL

Miniprep of plasmids
Tiago

All inoculums coming from 10/06 transformed colonies and yesterday's transformation with NEB cells were miniprepped.

Batch	*Plasmid*	*Concentration (ng/ul)*
NEB cells	pSB1C3 + Promoter	63,2
NEB cells	pSB1C3 + Resistance	18,8
NEB cells	pSB1C3 + Terminator	9,7
	Negative	6,7
On-day cells	pSB1C3 + Promoter	16,1
On-day cells	pSB1C3 + Resistance	19,0
On-day cells	pSB1C3 + Terminator	3,7

----------10/12----------

Digestion of plasmids
Brayan

Item	**pSB1C3 + Promoter**	**Rest of plasmids**
Cutsmart	1,5 ul	2,5 ul
XbaI	0,5 ul	2.0 ul
SpeI	0,5 ul	2.0 ul
Vector	10 ul	30 ul
Total	**15 ul ~**	**40 ul ~**

PCR of mCherry, 3' cassette

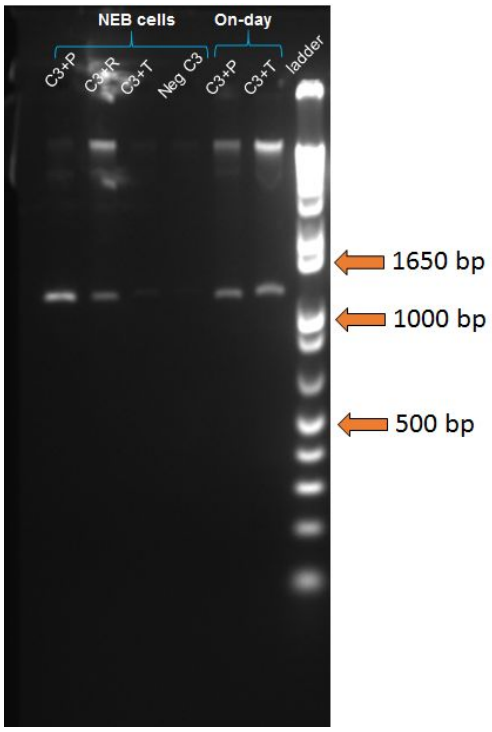
Brayan

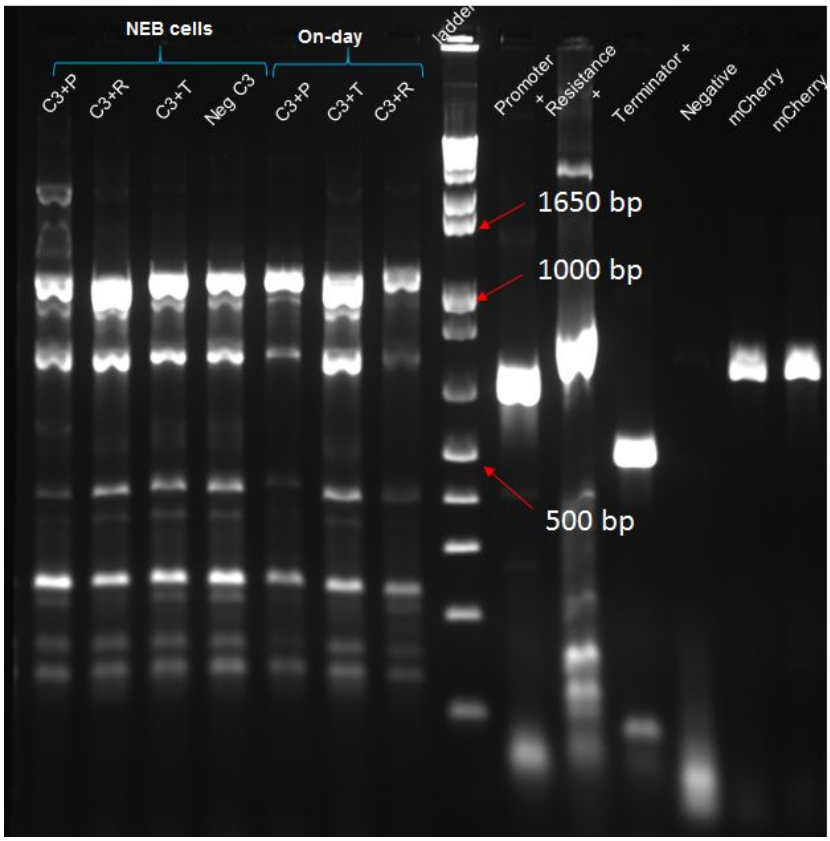
It was prepared five tubes for mCherry and four for 3’ cassette

Item	Volume for 1 reaction
Q5 master mix 2X (NEB)	12.5 µL
Forward primer	1,25 µL
Reverse primer	1,25 µL
Template DNA	1,0 µL
H20	*Qsf 25.0 µL
Total	25.0 µL

*qsf = quantity sufficient for

Gel electrophoresis of Colony PCR and digested plasmids
Brayan





Digestion of linearized and circular pSB1C3 and mCherry
Brayan

Item	**pSB1C3 + RFP**	**pSB1C3 + RFP**	**pSB1C3 + RFP**	**Linearized pSB1C3 + RFP**	**mCherry**	**mCherry**
Cutsmart	5	5	5	1	6	6
XbaI	1,5	1,5	1,0	0,5	1,5	1,5
SpeI	1,5	1,5	1,0	0,5	1,5	1,5
Vector	40	40	38	8	50	50
Total	**50 µL**	**50 µL**	**50 µL**	**10 µL**	**60 µL**	**60 µL**

----------10/14----------

Ligation of pSB1C3 to expression vector units
Brayan

Previous digested plasmids and PCR products were purified.
pSB1C3 gel purified = 6,6 ng/uL
pSB1C3 column purified = 10 ng/uL
mCherry = 94,8 ng/uL
Already double-digested expression vector units (Promoter, Resistance and Terminator) were used for this purpose.

pSB1C3 gel purified	Promoter	Resistance	Terminator	mCherry	Negative
Buffer 10X	1	1	1	1	1
T4 ligase	0,5	0,5	0,5	0,5	0,5
Vector	5,5	5,5	5,5	5,5	5,5
Insert	0,5	0,5	0,5	1,0	-
Water	2,5	2,5	2,5	2,0	3,5
pSB1C3 linearized					
Buffer 10X	1	1	1	1	1
T4 ligase	0,5	0,5	0,5	0,5	0,5
Vector	5	5	5	5	5
Insert	0,5	0,5	0,5	1	-
Water	3,0	3,0	3,0	2,5	4,0

Transformation of fresh ligated products
Brayan

All recent ligations underwent transformation with NEB cells. 5 dishes (1 per expression vector unit) were prepared for each backbone: C3 gel purified and C3 column purified.

----------10/15----------

Microalgae colony PCR

Transformant cells from 09/23 were transferred to a 96-well plate. Each well contained 200 uL of TAP medium and was filled with one colony. After 3 to 4 days, each well containing growing microalgae was picked and “imprinted” in a new TAP dish for colony PCR.

Colonies re-grew in this new TAP dish, and from these colonies, PCR was conducted.
Based on fluorescence and kinetic data gathered from previous days, top 20 of positive colonies were selected to amplify mCherry. They were splited between X7 and Q5 polymerase for PCR reactions.

Colonies: **A2, A11, B1, B2, B3, B6, B11, C3, C4, C11, D1, D2, E1, E2, E3, E5, F9, G2, G3, G9, G12**

Item	**Volume for 1 reaction**
Q5 master mix 2X (NEB)	6,25 µL
Forward primer	0,625 µL
Reverse primer	0,625 µL
Template DNA	1,0 µL
H20	*Qsf 12.5 µL
Total	12.5 µL

Colonies: **E3, C3, G12, B2, B11, E5**

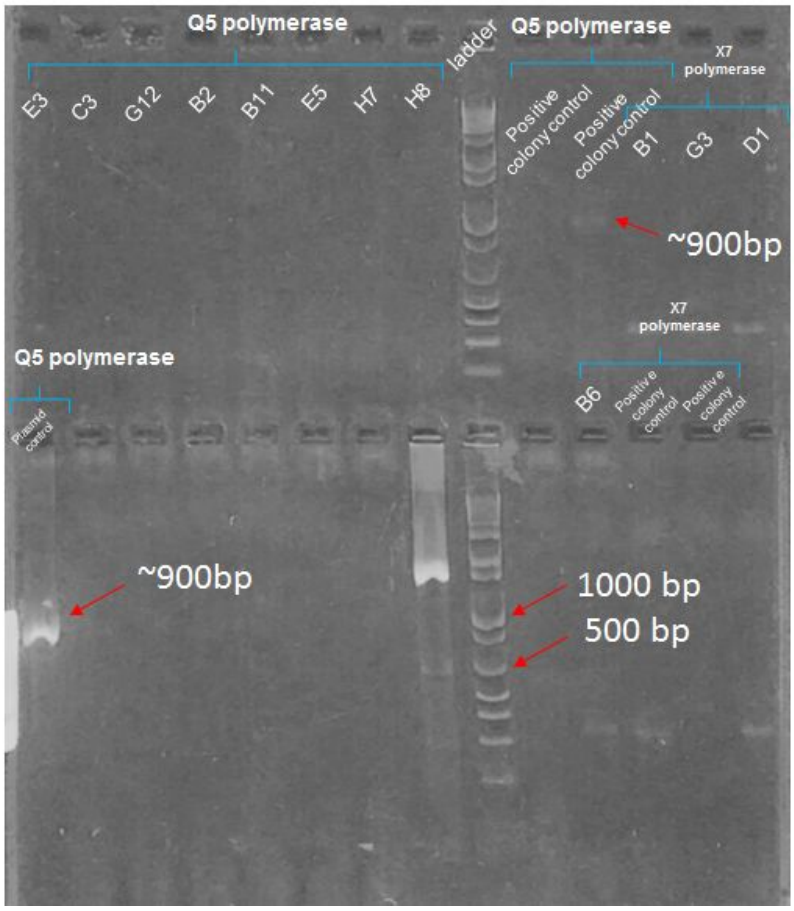
Rest of colonies were also tested with home-made X7 polymerase

Item	**Volume for 1 reaction**
Buffer GC	2,5
Betaine	2,5
Forward primer	0,625 µL
Reverse primer	0,625 µL
Polymerase	1,0 µL
dNTPs	0,625

Template DNA	1,0 µL
H20	*Qsf 12.5 µL
Total	12.5 µL

Colonies: **A2, A11, B1, B3, B6, C4, C11, D1, D2, E1, E2, F9, G2, G3, G9**

Positive colony PCR control from a different plate was also used. Positive plasmid control was also run.



Result: 1 out the 2 Positive colony control was amplified (Lane 11, red arrow). Rest of reactions does not show bands. It's strongly recommended to repeat PCR due to complexity of microalgae colony PCR

----------10/16----------

Colony PCR of transformant bacterias

10 reactions were prepared for each backbone with Q5 polymerase, whilst 15 reactions were prepared with X7 polymerase.

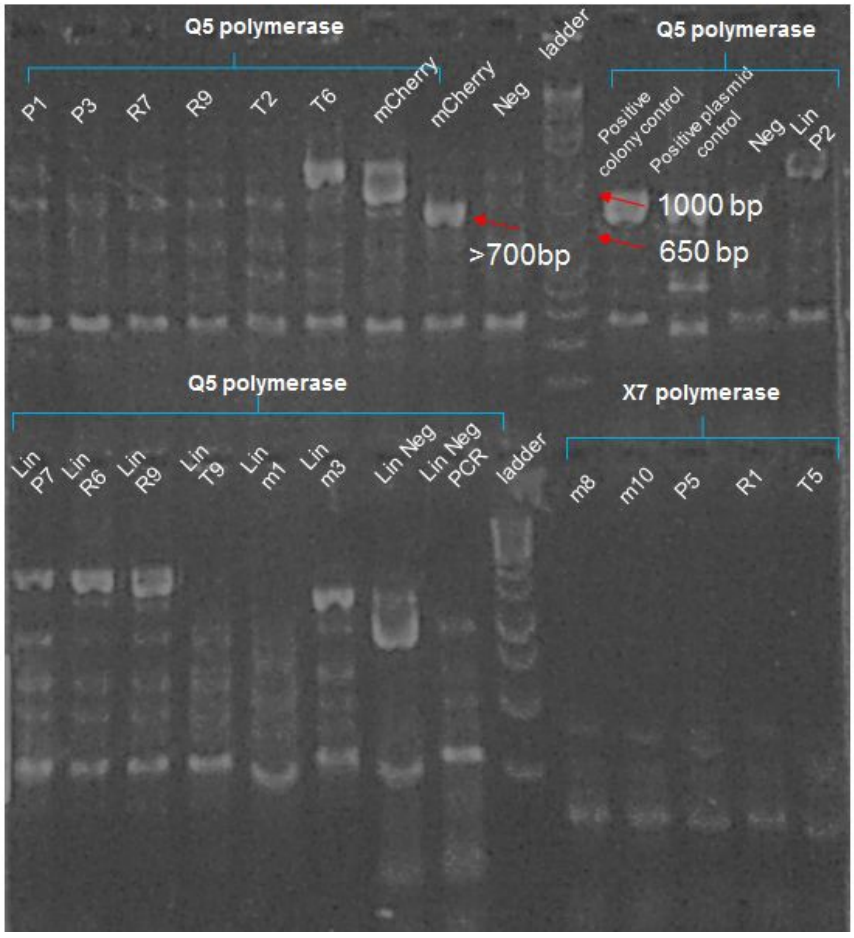
Item	**Volume for 1 reaction**
Q5 master mix 2X (NEB)	6,25 µL
Forward primer	0,625 µL
Reverse primer	0,625 µL
Template DNA	1,0 µL
H20	*Qsf 12.5 µL
Total	12.5 µL

pSB1C3 gel purified. Colonies' names: **P1, P3, R7, R9, T2, T6, m1, m3, -6, negative reaction**
pSB1C3 column purified. Colonies' names: **Lin P2, Lin P7, Lin R6, Lin R9, Lin T3, Lin T9, Lin m1, Lin m3, Lin Neg -2, Lin neg PCR**

Rest of colonies were also tested with home-made X7 polymerase

Item	**Volume for 1 reaction**
Buffer GC	2,5 µL
Betaine	2,5 µL
Forward primer	0,625 µL
Reverse primer	0,625 µL
Polymerase	1,0 µL
dNTPs	0,625
Template DNA	1,0 µL
H20	*Qsf 12.5 µL
Total	12.5 µL

pSB1C3 gel purified. Colonies' names: **P4, P5, R1, R8, T5, T8, m8, m10, -2, -4 negative reaction**
pSB1C3 column purified. Colonies' names: **Lin P3, Lin P9, Lin R3, Lin R7, Lin T5, Lin T6, Lin m4, Lin m7, Lin Neg -3, Lin Neg -6, Lin neg PCR**



Results: Positive colony and positive plasmid control strongly suggest that Lane 8 (mCherry colony m3) may bear cloned mCherry unit. This gleam of hope refill our will!